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Identification and Characterization by Antisense Oligonucleotides of Exon and Intron Sequences Required for Splicing

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Certain thalassemic human β-globin pre-mRNAs carry mutations that generate aberrant splice sites and/or activate cryptic splice sites, providing a convenient and clinically relevant system to study splice site selection. Antisense 2′-*O*-methyl oligoribonucleotides were used to block a number of sequences in these pre-mRNAs and were tested for their ability to inhibit splicing in vitro or to affect the ratio between aberrantly and correctly spliced products. By this approach, it was found that (i) up to 19 nucleotides upstream from the branch point adenosine are involved in proper recognition and functioning of the branch point sequence; (ii) whereas at least 25 nucleotides of exon sequences at both 3′ and 5′ ends are required for splicing, this requirement does not extend past the 5′ splice site sequence of the intron; and (iii) improving the 5′ splice site of the internal exon to match the consensus sequence strongly decreases the accessibility of the upstream 3′ splice site to antisense 2′-*O*-methyl oligoribonucleotides. This result most likely reflects changes in the strength of interactions near the 3′ splice site in response to improvement of the 5′ splice site and further supports the existence of communication between these sites across the exon.

Pre-mRNA splicing takes place within a large ribonucleoprotein complex termed the spliceosome. The specificity and accuracy of splicing are determined by the interactions of small nuclear ribonucleoprotein particles and protein components of the spliceosome with a number of pre-mRNA sequence elements in pre-mRNA, such as the branch point sequence, the polypyrimidine tract, and the 3′ and 5′ splice sites (reviewed in references 15, 21, and 29). In addition, exon sequences seem to contribute to the specificity of splicing (references 35, 41, and 49 and references therein). However, besides identification of a regulatory element in the female-specific exon of the doublesex pre-mRNA (17, 19, 30) and characterization of purine-rich motifs in exons from some other spliced transcripts (7, 12, 27, 45, 47, 49, 51), the involvement of exon sequences in splicing remains unclear.

In this work, we have used antisense 2′-*O*-methyl oligoribonucleotides (see reference 43 for a review) to study the function of several intron and exon sequences in pre-mRNA splicing. This approach stems from our recent report which showed that the binding of 2′-*O*-methyl oligoribonucleotides to the branch point or aberrant splice sites leads to the restoration of correct in vitro splicing of mutated β-globin pre-mRNAs identified in individuals with various forms of β-thalassemia (11). These oligonucleotides form strong duplexes with RNA which are resistant to RNase H and RNA unwinding activities. In consequence, they remain stably associated with the complementary regions in RNA, efficiently inhibiting the function of the targeted sequences. Antisense 2′-*O*-methyl oligoribonucleotides were originally used as sequence-specific probes to study the structure of small nuclear ribonucleoproteins and their interactions with the pre-mRNA substrate (43). They were also used to search for novel *cis*-acting sequence elements and possible scanning mechanisms during the splicing reaction (28).

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We have targeted these oligonucleotides against human β-globin pre-mRNAs carrying mutations responsible for a number of β-globin thalassemia variants. The mutations generate new splice sites and/or activate cryptic ones, leading to aberrant splicing pathways (reviewed in reference 40). In the experiments described below, we took advantage of the fact that changes in the accumulation of the correctly spliced products relative to that of the aberrant ones provide a sensitive and internally controlled assay for monitoring interference with the activity of the sequence targeted by the antisense oligonucleotide. Using this approach, we have found that sequences upstream from the conserved branch point sequence as well as those within the exons are required for splicing and play a role in splice site selection. The experiments also showed that improving the 5′ splice site of the internal exon affects interactions at the upstream 3′ splice site.

MATERIALS AND METHODS

Plasmid construction. Previously described pSP64HβΔ6 (22) and its thalassemic derivatives cloned in the pSP64 vector were used in all studies. The pIVS1-β^{110} clone, carrying an A-to-G mutation in position 110 of the first intron, was constructed by subcloning an appropriate fragment from the original thalassemic clone (14a). The pIVS2-β^{705} clone was obtained by introducing a T-to-G mutation at position 705 of the large β-globin intron in the pIVS2 clone, as previously described (11, 25). A C-to-T mutation at position 654 and a TA-to-GT mutation at positions 657 and 658 of the large β-globin intron in the pIVS2 clone were introduced to create pIVS2-β^{654} and pIVS2-β^{654con} clones, respectively. The structures of all mutated constructs were confirmed by sequencing.

In vitro transcription and splicing. ^{32}P-labeled transcripts were synthesized as previously described (20) with SP6 RNA polymerase (Promega) and DNA templates cleaved within the second exon at either the *Bam*HI site (clones HβΔ6 and β^{110}), the *Pvu*II site (clone β^{705}), or the *Ase*I site (clones pIVS2, β^{654}, and β^{654con}). The preparation of nuclear extract from HeLa

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)	% GC
BPa	GUCAGUGCCUAUCA	50
BPb	GUGCCUAUCAGAAA	43
BPc	CCUAUCAGAAACCC	50
BPc/18	CCUAUCAGAAACCCAAGA	44
BPd	AUCAGAAACCCAAG	43
BPe	AAACCCAAGAGUCU	43
BPf	CAAGAGUCUUCUCU	43
Exa	ACCAGCAGCCUAAG	57
Exb	AGGGUAGACCACCA	57
Exc	UCUGGGUCCAAGGG	64
Exd	UCAAAGAACCUCUG	43
3′ss 579/14	UAUUGCCCUGAAAG	43
3′ss 579/18	CAUUAUUGCCCUGAAAGA	39
ExU1	GUAUCAUUAUUGCC	36
ExU2	UACAUUGUAUCAUU	21
ExU3	AGAGGCAUGAUACA	43
ExU4	AAUGGUGCAAAGAG	43
ExU5	UAUUCUUUAGAAUG	21
ExD1	CAGAAAUAUUUAUA	14
ExD2	AAUUUAUAUGCAGA	21
5′ss 705	CCUCUUACCUCAGUUAC	47
Int1	UAUUAGCAAUAUGA	21
Int2	UUGUAGCUGCUAUU	36
5′ss 652/18	GCUAUUACCUUAACCCAG	44
5′ss 652con/18	GCACUUACCUUAACCCAG	50
5′ss 652con/14	CACUUACCUUAACC	43

cells and in vitro splicing were performed as previously described (11, 22).

Synthesis and purification of oligonucleotides. 2′-*O*-methylribonucleoside phosphoramidates (Glen Research, Sterling, Va.) were used for oligonucleotide synthesis in an Applied Biosystems synthesizer at the Lineberger Comprehensive Cancer Center. Oligonucleotides were purified by thin-layer chromatography (SurePure kit; U.S. Biochemicals), and their concentrations were determined spectrophotometrically at 260 nm. To ascertain the quality of oligonucleotides, the 5′-^{32}P-labeled compounds were analyzed by polyacrylamide gel electrophoresis.

Oligonucleotide treatment. In all experiments, 2′-*O*-methyl oligoribonucleotides were added to the reaction mixture together with the other components of the splicing reaction. The preannealing of oligonucleotides with transcript in the absence of nuclear extract has been shown not to increase their overall effect on splicing (11, 28). The extent of unspecific effects for each experiment was controlled by using oligonucleotides with no complementarity to the RNA substrate. Oligonucleotide 3′ss 579/14 was used to control unspecific effects during the splicing of pre-mRNAs containing the first β-globin intron, whereas oligonucleotide BPa was used during the splicing of pre-mRNAs containing the second intron. In some experiments, a mixture of randomly synthesized oligonucleotides was also used.

Sequences of oligonucleotides. The oligonucleotides used in this study are listed in 5′-to-3′ orientation in Table 1.

Data processing and analysis. All autoradiograms were captured by a DAGE MTI CCD72 video camera (DAGE, Michigan City, Ind.), and images were processed with NIH Image 1.47 and MacDraw Pro 1.0 software. The final figures were printed out on a Sony dye sublimation printer. Results were quantitated with NIH Image 1.47 software and expressed as percentages of correct product relative to the sums of correct and aberrant products. Values were adjusted to account for the higher number of ^{32}P-labeled C nucleotides in the longer, aberrantly spliced product. Averages obtained from several independent experiments and multiple exposures are presented.

RESULTS

Mapping the upstream boundary of the functional domain near the branch point sequence with β^{110} pre-mRNA substrate. A G-to-A point mutation at position 110 of the first intron in the β-globin gene (IVS1-β^{110}; Fig. 1B) creates a new 3′ splice site at position 109 in addition to the one located at position 130 of this intron. This mutation is responsible for a significant number of β-thalassemia cases in southeastern Europe, Cyprus, and Lebanon (40). The splicing of IVS1-β^{110} mutant pre-mRNA leads to predominant (approximately 90%) accumulation of a spliced product containing an additional 19 nucleotides of the intron sequence (Fig. 2A, lane 1). Remarkably, aberrant splicing of β^{110} pre-mRNA occurs via selection of the regular branch point at position 94 of the intron, whereas correct splicing (approximately 10% of the resultant mRNA) occurs via selection of the cryptic branch point at position 107 (54). As a result, mutations inactivating the regular branch point (38) or antisense oligonucleotides blocking it (11) stimulate the cryptic branch point and restore correct splicing in the β^{110} background.

In these studies, we used 2′-*O*-methyl oligoribonucleotides to determine the importance of sequences located upstream from the branch point region in the function of this splicing element. We have designed a series of 14-mers complementary to the region extending up to 32 nucleotides upstream from the branch point sequence (Fig. 1B). The ability of oligonucleotides to inhibit splicing at the aberrant 3′ splice site and at the same time to promote splicing at the correct 3′ splice site was taken as the measure of the function of the upstream sequences during the splicing of β^{110} pre-mRNA (11). Note that a concomitant switch in selection between the regular and cryptic branch points can be directly determined on autoradiograms because of the variable mobility of the corresponding lariats (54).

The results of in vitro splicing of β^{110} pre-mRNA carried out in the presence of 0.5, 2.0, and 10.0 μM oligonucleotide BPa, blocking all seven nucleotides of the branch point sequence, are shown in Fig. 2A. Consistent with the results of a previous report (11), the antisense 2′-*O*-methyl oligoribonucleotide targeted to this site leads to a change in the ratio between correct and aberrant products. A splicing reaction carried out at a 2 μM concentration of this oligonucleotide results in the accumulation of approximately 55% of the correct product (Fig. 2A, lane 3; see Fig. 2C for quantitation). Although 10 μM oligonucleotide BPa results in a marked decrease of the overall efficiency of the splicing reaction, it does not significantly modify the ratio between correct and aberrant products achieved at a 2 μM concentration (Fig. 2A, lane 4).

Figure 2B demonstrates the results of an in vitro splicing reaction of β^{110} pre-mRNA carried out in the presence of a 2 μM concentration of oligonucleotides directed upstream from the branch point adenosine. Similar to BPa, oligonucleotides BPb, -c, and -d restore correct splicing to 50 to 65% (Fig. 2B, lanes 2 to 4, respectively, and C). Note that oligonucleotides BPc and BPd hybridize immediately outside the conserved branch point sequence and four nucleotides upstream from this element, respectively. While oligonucleotide BPe, hybridized 13 nucleotides upstream from the branch point adenosine, still has some effect on the splicing pathway, oligonucleotide

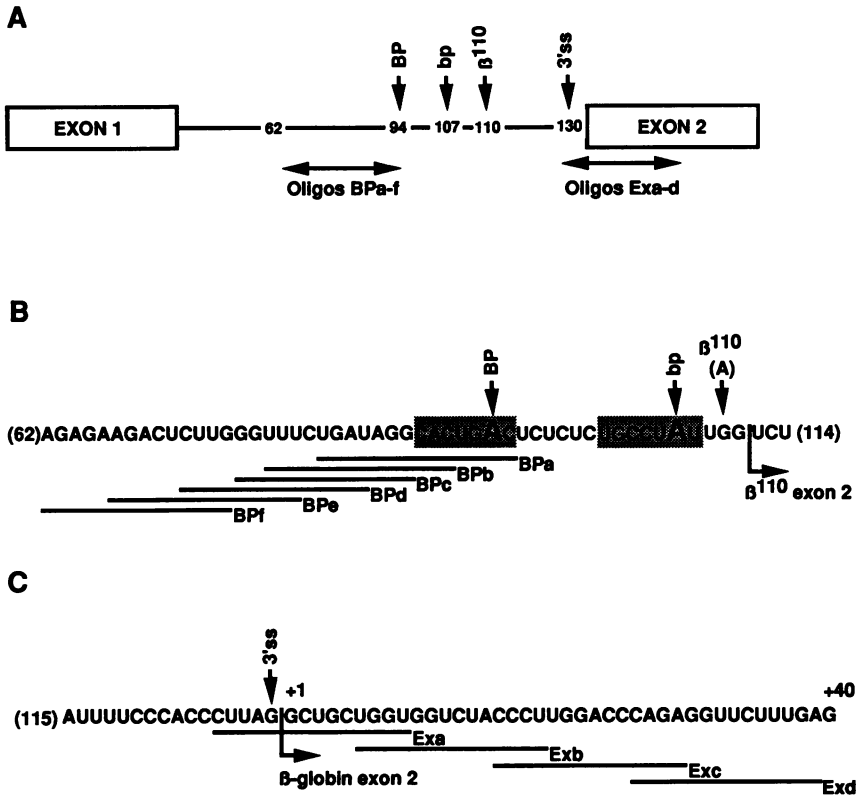

FIG. 1. (A) Structure of human β-globin pre-mRNA (not drawn to scale) containing the first intron (the line) and two flanking exons (boxes). The positions of the regular (BP) and cryptic (bp) branch point adenosines, β^{110} mutation, and the 3′ splice site (3′ss) are shown. The regions of pre-mRNA used as targets for 2′-*O*-methyl oligoribonucleotides are indicated by double-headed arrows. Exon 1 contains 154 nucleotides, and exon 2 contains 210 nucleotides. The exact sequence positions of oligonucleotides BPa to BPf (B) and Exa to Exd (C) are shown. The regular and cryptic branch point sequences (shaded boxes) and the aberrant (β^{110}) and correct 3′ splice sites are indicated.

BPf, targeted 5 nucleotides further upstream, seems to be ineffective. Consistently, oligonucleotide BPf, in contrast to the other probes tested, does not stimulate the formation of the lariat intermediate at the branch point adenosine at position 107, as indicated by the lack of a band migrating to the top of the gel (Fig. 2B, lane 6).

The involvement of exon sequences in the splicing of β^{110} pre-mRNA. To survey other regions of β-globin pre-mRNA for the existence of functional domains involved in splicing, we designed 14-mers complementary to the correct junction between the first intron and the second exon (oligonucleotide Exa) or to sequences extending downstream into the second exon (oligonucleotides Exb to -d) (Fig. 1C). For HβΔ6 pre-mRNA, oligonucleotide Exa partially overlaps with the intron sequence; in the case of β^{110} substrate, the same oligonucleotide has its target entirely within the aberrantly spliced second exon. Although the upstream end of the duplex formed between this oligonucleotide and its target in the β^{110} transcript is separated from the predominantly used aberrant 3′ splice site by 14 nucleotides, Exa oligonucleotide at a 2 μM concentration fully blocks splicing (Fig. 3, lane 3). This is in agreement with the observation that mutations disrupting the AG dinucleotide of the normal 3′ splice site inhibit the use of the aberrant splice site in β^{110} pre-mRNA (23, 54). An inhibitory effect on splicing is also observed in the presence of oligonucleotides Exb and Exc, which hybridize further downstream into the second exon, and, albeit to a lesser extent, in the presence of the most distal oligonucleotide, Exd, which hybridizes 44 nucleotides downstream from the β^{110} 3′ splice site (Fig. 3, lanes 4 to 6). The control noncomplementary oligonucleotide does not significantly affect the efficiency of the splicing reaction (Fig. 3, lane 7). A pattern of inhibition similar to that described above was obtained when each of the oligonucleotides Exa, -b, -c and -d was added to the splicing reaction with normal β-globin pre-mRNA (data not shown).

The involvement of exon sequences in the splicing of the β^{705}-globin pre-mRNA. The results of Zhuang and Weiner (54) suggested that the splicing of β^{110} pre-mRNA may represent
a special case in which the AG dinucleotide located inside the exon is required for splicing at the upstream 3′ splice site. To confirm that exon involvement in splicing is not limited to only one type of substrate, we analyzed the effects of a series of antisense oligonucleotides (Fig. 4B and C) on the splicing of thalassemic β^{705} pre-mRNA. A T-to-G mutation at position 705 of the large β-globin intron creates an additional 5′ splice site and activates a cryptic 3′ splice site at position 579 of this intron (8). The incorrect splicing pathway resulting from the utilization of both splice sites leads to the incorporation of nucleotides 580 to 705 of the intron into the spliced product and the accumulation of significant amounts of the 577-nucleotide aberrant RNA in addition to the correct product (Fig. 4A). Changes in the ratio between correct and aberrant products provide a sensitive method for measuring the sequence-specific effects of the oligonucleotides used.

Figure 5A shows the complete pattern of in vitro splicing of the β^{705} substrate. Quantitative analysis indicates that the ratio

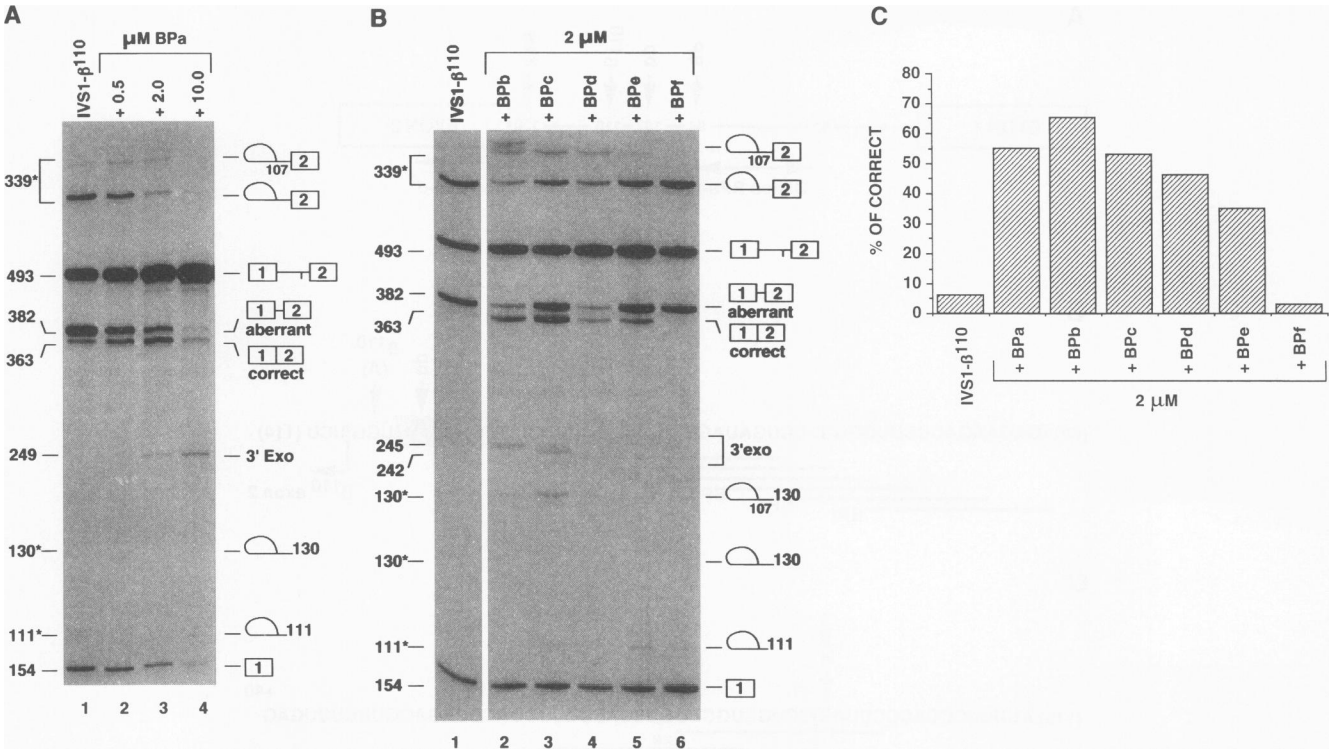

FIG. 2. (A) Change in the ratio between correct and aberrant products during the splicing of β^{110} pre-mRNA in the presence of oligonucleotide BPa, targeted to the branch point sequence. The concentration of oligonucleotide is shown at the top of each lane. The control splicing pattern for β^{110} pre-mRNA is shown in lane 1. Bands at the top of the autoradiogram correspond to the lariat-exon 2 intermediate, formed at the cryptic branch point at nucleotide 107 (upper) and at the regular adenosine at nucleotide 94 (lower). The 249-nucleotide RNA product visible in lanes 3 and 4 is generated by endogenous 3′-to-5′ exonuclease (3′ Exo) blocked at the site of duplex formation (reference 11 and unpublished data). The structures of splicing products and intermediates are shown on the right, and their sizes (in nucleotides) are on the left. An asterisk denotes the unusual mobility of the corresponding lariat-containing RNA species. The same designations are used in subsequent figures. (B) Effects of oligonucleotides BPb to BPf, targeted upstream from the branch point adenosine, on the splicing of β^{110} pre-mRNA. (C) Quantitative analysis of the reactions carried out in the presence of 2 μM oligonucleotides. Data were calculated as the percentages of the correct product in overall splicing (see Materials and Methods).

of correct to aberrant products is approximately 1:1 (Fig. 5B, lane 1). Aberrant splicing is almost completely suppressed in the presence of 2 μM 14-mer oligonucleotides targeted either to the 3′ splice site (oligonucleotide 3′ss 579; Fig. 5B, lane 2) or to the exon sequence immediately downstream from this site (oligonucleotide ExU1; Fig. 5B, lane 3). Oligonucleotides targeted to exon sequences between nucleotides 7 and 20 (ExU2), 16 and 30 (ExU3), and 27 and 40 (ExU4) also block aberrant splicing, although not as efficiently as the first two probes mentioned immediately above (Fig. 5B, lanes 4 to 6, respectively). The most distant probe (oligonucleotide ExU5), complementary to nucleotides 37 to 50 of the exon, has only a limited effect on the aberrant splicing pathway of β^{705} pre-mRNA and delimits the downstream boundary of the exon region that plays a role in splicing (Fig. 5B, lane 7).

The involvement of exon sequences in splice site selection was further confirmed by using oligonucleotides ExD1 and ExD2, which hybridize to a region near the downstream end of the exon and lead to virtually full inhibition of the aberrant splicing pathway (Fig. 5C, lanes 2 and 3). As expected (11), a strong inhibitory effect is also mediated by oligonucleotide 5′ss 705, targeted to the exon-intron junction (Fig. 5C, lane 4). Interestingly, oligonucleotides Int1 and Int2, targeted to regions located entirely within the intron and almost immediately downstream from the 5′ splice site, have little, if any, effect on aberrant splicing (Fig. 5C, lanes 5 and 6).

Testing the accessibility of 3′ and 5′ splice sites to 2′-*O*-methyl oligonucleotides in β^{654} and β^{654con} pre-mRNAs. It has been shown for a variety of pre-mRNA substrates that mutations within the 5′ splice site of an internal exon affect the splicing of the upstream intron (1, 10, 26, 31, 46). This effect suggests the existence of a certain type of communication between splice sites across the exon and argues in favor of the exon definition model (36). In order to determine whether an improvement of the 5′ splice site to the consensus sequence increases interactions near the upstream 3′ splice site flanking the internal exon, we tested the accessibility of these sites to antisense oligonucleotides in β^{654}-globin pre-mRNA and its modified version, β^{654con}. β^{654} represents another thalassemic mutation which occurs in the human β-globin gene and results in the generation of aberrantly spliced product, containing part of the second intron (5). The C-to-T mutation at nucleotide 654 of the intron creates a new 5′ splice site at position 652 (AG/GUAAUA), activates a cryptic 3′ splice at position 579, and results in the generation of a 73-nucleotide aberrant exon (Fig. 6). We improved the aberrant 5′ splice site at position 652 by two point mutations, including a highly conserved G at position +5, thereby creating the consensus element AG/GUAAGU (substituted nucleotides are underlined). These

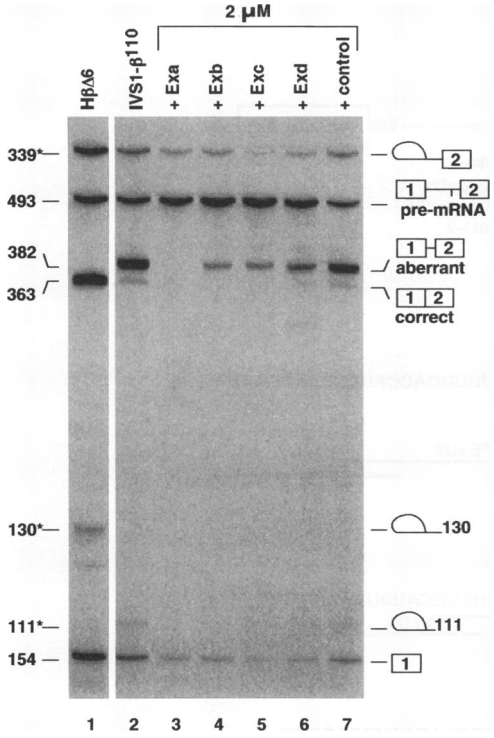

FIG. 3. Inhibition of splicing by oligonucleotides targeted to aberrant exon 2. The splicing of β^{110}-globin pre-mRNA in the presence of 2 μM oligonucleotides Exa to Exd is shown in lanes 3 to 6, respectively. The splicing reactions of HβΔ6 and β^{110} pre-mRNAs with no oligonucleotides are shown in lanes 1 and 2, respectively. Lane 7, the splicing of β^{110} pre-mRNA in the presence of noncomplementary oligonucleotide (control) (see Materials and Methods).

changes increase the base pairing of the 5′ splice site to the 5′ end of U1 small nuclear RNA and result in more efficient selection of this site (32, 52, 53). The full splicing patterns for both the original (β^{654}) and improved (β^{654con}) pre-mRNAs are shown in Fig. 7 (lanes 2 and 3, respectively). In addition, the pattern for wild-type pre-mRNA, containing no mutation in the intron, is presented in lane 1 of Fig. 7. As expected, creation of the perfect 5′ splice site at position 652 leads to complete inclusion of the aberrant exon.

Consistent with the results of a previous report (11), 18-mer oligonucleotides targeted to either 3′ or 5′ splice sites in β^{654} pre-mRNA inhibit the aberrant splicing pathway with similar efficiency. At 0.25 μM concentrations, both oligonucleotides lead to a substantial reduction in the amount of the aberrant product (Fig. 8A, lanes 2 and 5); at 0.75 μM concentrations, they lead to full restoration of the correct pathway (Fig. 8A, lanes 3 and 6). On the basis of these results, we conclude that in β^{654} pre-mRNA, 3′ and 5′ splice sites are equally accessible to antisense oligonucleotides. The results of a similar analysis with β^{654con} pre-mRNA are markedly different. An 18-mer targeted to the 5′ splice site restores correct splicing with an efficiency not drastically different from that in the splicing of β^{654} pre-mRNA (Fig. 8B, lane 5), in spite of the fact that a strong consensus 5′ splice site leads to exclusively aberrant splicing of β^{654con} pre-mRNA (Fig. 8B, lane 1). In contrast, an 18-mer targeted to the 3′ cryptic splice site that at 0.25 μM efficiently restored correct splicing of β^{654} pre-mRNA is inactive at this concentration when targeted to the same site in β^{654con} pre-mRNA (Fig. 8B, lane 2). Some effect becomes detectable at 0.75 μM (Fig. 8B, lane 3), and only at 2.5 μM is correct splicing fully restored (Fig. 8B, lane 4). These results demonstrate that the 3′ splice site in β^{654con} pre-mRNA is a much more resistant target for 2′-*O*-methyl oligoribonucleotides than the engineered consensus 5′ splice site.

The difference in relative accessibility between aberrant 3′ and 5′ splice sites in β^{654con} pre-mRNA is even more apparent when they are targeted with 14-mers. The oligonucleotide targeted to the 3′ splice site in β^{654con} is totally ineffective even at a 2.5 μM concentration, whereas the one targeted to the 5′ splice site restores 30% of correct splicing at a 0.25 μM concentration and 70% at a 2.5 μM concentration (Fig. 9A, lanes 4 and 5, respectively). The inactivity of the 14-mer targeted to the 3′ splice site does not result from any reason other than the low accessibility of the target site in β^{654con} pre-mRNA (for example, the formation of the secondary structure), since the same preparation of this oligonucleotide at a 0.25 μM concentration leads to 70% of the correct product (Fig. 9B, lane 2) and at a 2.5 μM concentration leads to virtually full inhibition of the aberrant splicing pathway of β^{654} pre-mRNA (Fig. 9B, lane 3). The control oligonucleotide tested with both pre-mRNAs at a 2.5 μM concentration does not affect the splicing reaction (Fig. 8B, lane 8, and 9B, lane 4).

DISCUSSION

To determine the upstream boundary of the functional domain near the branch point sequence, we used several antisense 2′-*O*-methyl oligoribonucleotides complementary to targets upstream from the regular branch point in β^{110} pre-mRNA. The results show that no more than 19 nucleotides upstream from the branch point adenosine are involved in the proper recognition and functioning of this splicing element. Previously reported data revealed several possible boundaries for the branch point domain, varying in length from 5 to 39 nucleotides upstream from the branch adenosine (3, 37). Since those results were based on RNase A and RNase T1 protection assays, the heterogeneity in the size of the protected region could result from either overdigestion or underdigestion by enzymes (37). Furthermore, since 2′-*O*-methyl oligoribonucleotides remain stably associated with target RNA and prevent interactions with splicing factors throughout the entire course of the splicing reaction, they offer an experimental approach different from that of the nuclease protection assay (3, 4, 24, 37) or oligonucleotide-mediated cleavage of RNA by RNase H (37, 39). The last two methods detect only those interactions between RNA and splicing factors that exist at the time of nuclease treatment.

Oligonucleotides BPa and BPb are targeted to the regular branch point and at the same time to 12 and 16 nucleotides upstream from the cryptic branch point sequence, respectively. The binding of these oligonucleotides to pre-mRNA also seems to affect the function of this branch point and leads to the activation of yet another branch point (most likely the adenosine at position 115), as indicated by the generation of an additional slowly migrating intermediate at the top of the gel (Fig. 2A, lane 3, and B, lane 2). More distant oligonucleotide probes (BPc to Bpe), effective in blocking the regular branch point, have their targets beyond the cryptic branch point domain and do not affect its function. This observation additionally supports the postulated extent of interactions upstream from the branch point sequence. The mapping of interactions from the downstream side of the branch point sequence by using the same approach was not possible, presumably because of the proximity of the polypyrimidine tract

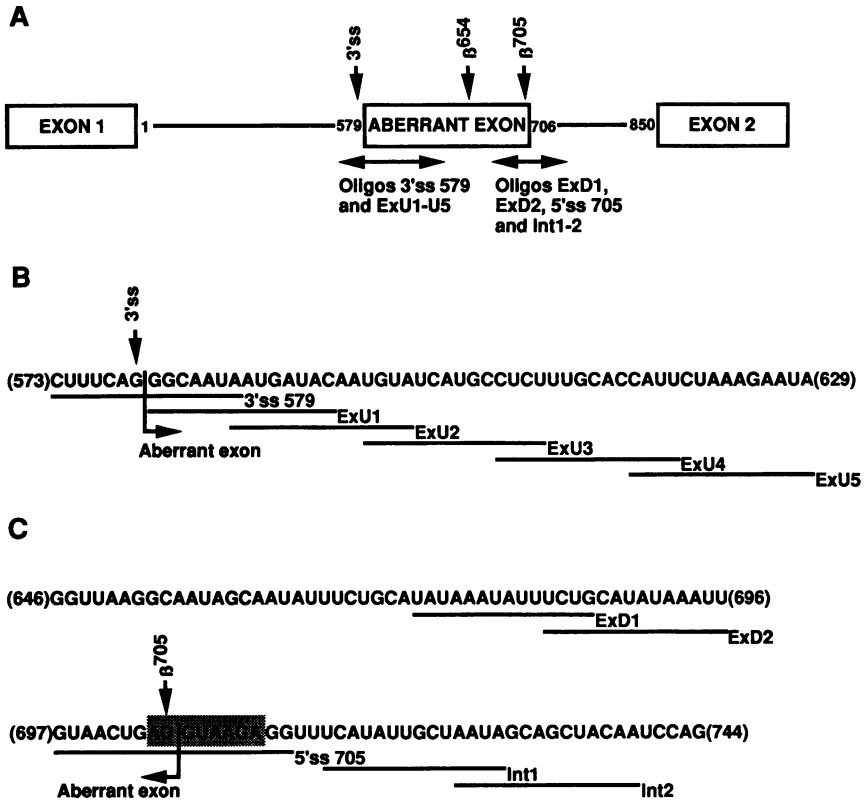

FIG. 4. (A) Structure of β^{705} pre-mRNA. This RNA contains the 126-nucleotide aberrant exon created by a mutation at position 705 of the 850-nucleotide β-globin intron 2. The position of the β^{654} mutation (see also Fig. 6) is indicated above the aberrant exon. The correct exon 1 contains 222 nucleotides; exon 2 contains 229 nucleotides. Aberrant introns 1 and 2 contain 579 and 145 nucleotides, respectively. The regions of pre-mRNA used as targets for 2′-*O*-methyl oligoribonucleotides are indicated by double-headed arrows. (B and C) These oligoribonucleotides are shown in more detail as bars below the intron sequence. The 5′ splice site at position 705 is indicated by the shaded box.

and the cryptic branch point. Targeting oligonucleotides to this region inhibited both splicing pathways simultaneously (reference 11 and data not shown).

Although it has been known that both the sequences (6, 12, 14, 35, 41, 49, 51) and the lengths (2, 9, 13, 14, 34, 44, 48, 51) of exons are important determinants in the mechanism of splice site selection, the details of their involvement are not well understood. We have presented here a method for probing exon sequences with 2′-*O*-methyl oligoribonucleotides in search of the signals required for splice site selection. The existence of one such signal, the AG dinucleotide of the correct 3′ splice site originally identified by genetic analysis of the β^{110} mutant (23, 54), was confirmed here by masking it with the antisense oligonucleotide Exa. Interestingly, nonconserved regions of the exon located further downstream, beyond the AG dinucleotide, also seem to play an important role in splicing. This is demonstrated by the inhibitory effects of oligonucleotides Exb and -c. The effect of oligonucleotide Exd, which hybridized to nucleotides 26 to 40 of the exon, was not as pronounced, suggesting that the boundary of exon sequences important for splicing lies within this region. This may explain why in another study (28) an oligonucleotide that was two nucleotides shorter and targeted to the same region failed to show any inhibitory effect.

Although splicing inhibition was reproducibly observed in several independent experiments, the possibility that the decreased efficiency of splicing in the presence of oligonucleotides Exa, -b, -c, and -d was in fact caused by their unspecific effects or resulted from normal variabilities between splicing reactions could not be ruled out. An internally controlled system was provided by β^{705} pre-mRNA. Oligonucleotides ExU1 to -4 targeted to the aberrant exon in this substrate, resulted in predominant accumulation of the correct product instead of an overall inhibition of splicing, confirming the specificity of the observed effect. Since oligonucleotide ExU5 was not able to switch splicing pathways, we conclude that the essential region of the aberrant exon is limited to 27 to 36 nucleotides downstream from the 3′ splice site (upstream limits for ExU4 and ExU5 targets, respectively), i.e., similar to the analogous region in HβΔ6 pre-mRNA. The region essential for splicing upstream from the 5′ splice site does not appear to be shorter than 20 nucleotides and could be as extensive as the one at the other end of the exon. In contrast, the upstream region of the intron required for splicing is limited almost exclusively to the aberrant 5′ splice site. Although the target for oligonucleotide Int1 is separated from the 3′ flanking nucleotide of this splice site by only four nucleotides, the oligonucleotide did not promote correct splicing. This result argues against the possibility that the binding of 2′-*O*-methyl oligoribonucleotides to RNA generates long-range changes in the required structure of the substrate pre-mRNA and prevents the association of splicing factors at locations distant from the site of duplex formation. Such a remote effect would significantly decrease the accuracy of the mapping procedure described here by overes-

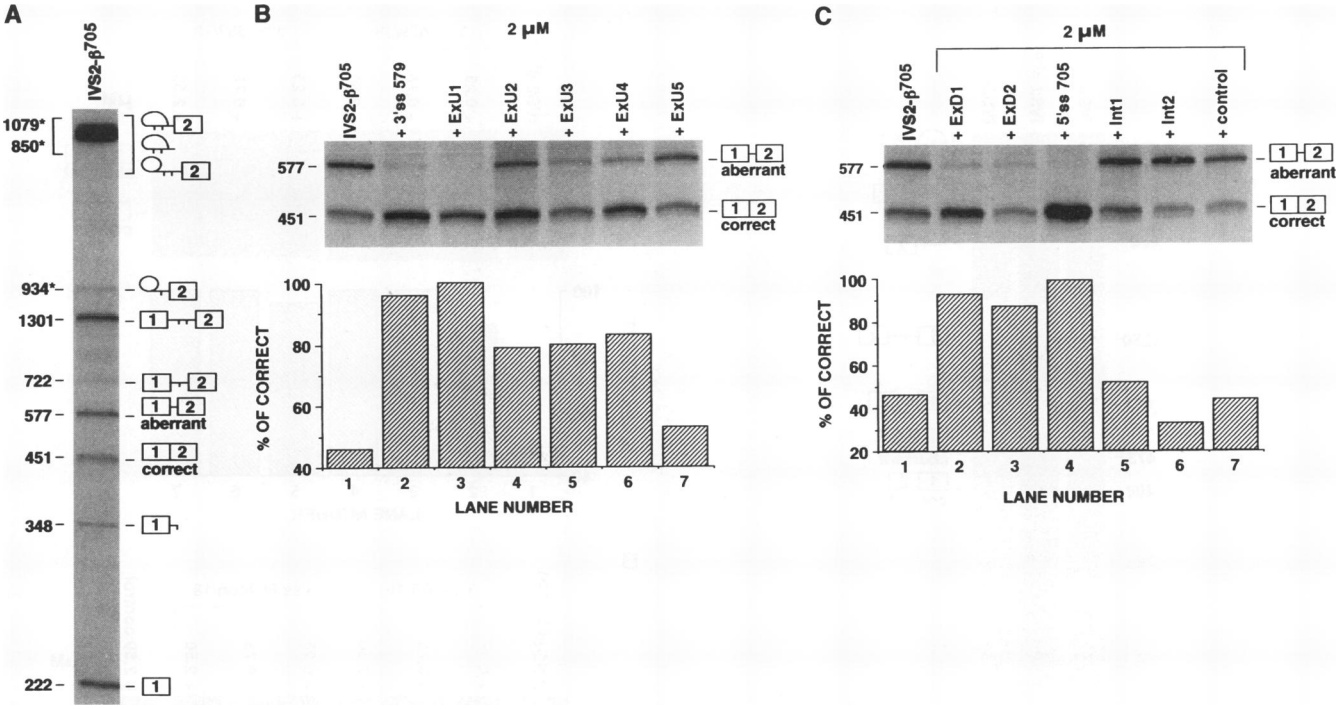

FIG. 5. (A) Complete pattern of products and intermediates generated during in vitro splicing of β^{705} pre-mRNA. The reaction proceeds by two pathways, leading to the accumulation of correctly and aberrantly spliced products. Large lariat structures at the top of the gel comigrate at the concentration of acrylamide used in this experiment (8%). The structures and sizes of all RNA species were determined previously (11) and are shown on the right and the left of the autoradiogram, respectively. Aberrant 3′ and 5′ splice sites are represented by short vertical lines in the intron. The same designations are used in subsequent figures. (B) Change in the ratio between correct and aberrant products by oligonucleotides 3′ss 579 and ExU1 to ExU5. The splicing reaction of β^{705} pre-mRNA was carried out in the presence of 2 μM oligonucleotides targeted to the aberrant 3′ splice site (oligonucleotide 3′ss 579 [lane 2]) and to upstream sequences of the aberrant exon (oligonucleotides ExU1 to ExU5 [lanes 3 to 7, respectively]). Lane 1, control splicing with no oligonucleotide. For simplicity, only the part of the autoradiogram which contains spliced products is shown here and in panel C. Quantitation of the results is shown below the autoradiogram. (C) Effects of oligonucleotides targeted to sequences near the aberrant 5′ splice site. The splicing of β^{705} pre-mRNA was carried out in the presence of oligonucleotides targeted to the downstream part of the aberrant exon (oligonucleotides ExD1 and ExD2 [lanes 2 and 3]), the 5′ splice site at position 705 (oligonucleotide 5′ss 705 [lane 4]), and adjacent intron sequences (oligonucleotides Int1 and Int2 [lanes 5 and 6]). Lane 7, splicing with noncomplementary oligonucleotide.

timating the actual boundaries of the domains involved in splicing.

Several lines of evidence show that the lack of effect for certain oligonucleotides is not due to their inherent inability to hybridize to the target sequence. A sequence comparison of the oligonucleotides tested in this work suggests that their

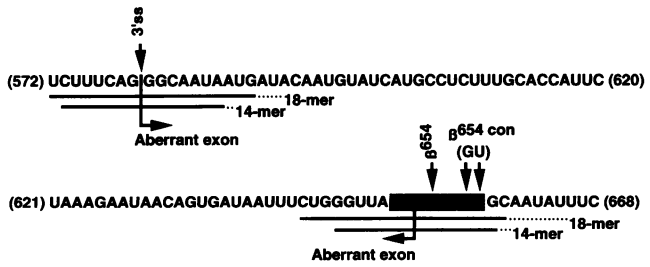

FIG. 6. Sequence of the 73-nucleotide aberrant exon in β^{654} pre-mRNA. The cryptic 3′ splice site at position 579 and the 5′ splice site (shaded box) created by the mutation at position 654 of β-globin intron 2 are indicated. The two-nucleotide mutation resulting in the generation of the consensus 5′ splice site β^{654con} is shown above the shaded box. The positions of 14- and 18-mer 2′-*O*-methyl oligoribonucleotides targeted to both splice sites are indicated below the sequence.

different compositions and hence potential variations in the strength of binding to target sequences did not contribute significantly to their differences in the ability to affect splicing. For example, oligonucleotides Int1 and Int2, with 21 and 36% GC content, respectively, are ineffective, although they are expected to anneal to complementary sequences on RNA with higher affinity than the effective oligonucleotides ExD1 (14% GC) and ExD2 (21% GC). Oligonucleotides BPb and BPf, with an identical GC content (48%), exert drastically different effects, consistent with their respective distances from the branch point sequence. Furthermore, one oligonucleotide (3′ss 579/14) affects splicing when targeted to the 3′ cryptic splice site sequence in β^{654} pre-mRNA but does not affect splicing if targeted to the same sequence in β^{654con} pre-mRNA. Finally, direct evidence is provided by the fact that oligonucleotides BPf and BPc, with very different effects on splicing, served equally efficiently as primers in a primer extension experiment (data not shown).

The results presented in this work demonstrate that approximately 50 to 55 nucleotides of exon sequence are required in vitro for aberrant splicing of β^{705} pre-mRNA substrate. This correlates well with the minimal size of exons commonly occurring in nature. As indicated by statistical analysis, internal exons containing less than 50 nucleotides are rare and constitute no more than 4% of all exons identified in vertebrates

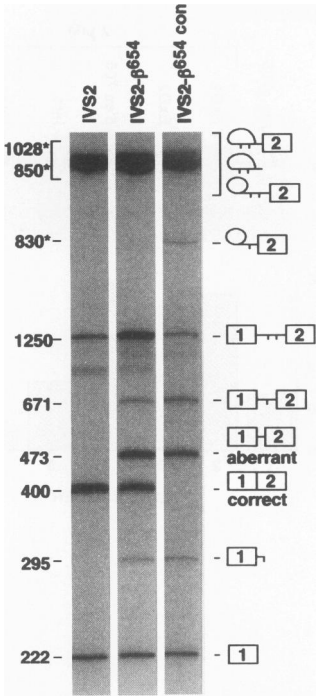

FIG. 7. Complete pattern of products and intermediates generated during in vitro splicing of IVS2, β^{654}, and β^{654con} pre-mRNAs. IVS2 represents the wild-type substrate, which follows a simple splicing pathway, yielding the 400-nucleotide correct product (composed of exons 1 and 2). The splicing of β^{654} thalassemic pre-mRNA results in the generation of aberrant mRNA containing an extra 73 nucleotides of intron sequence in addition to the correct product. The splicing of β^{654con} pre-mRNA, because of mutations improving the 5′ splice site at position 652, results in the generation of only the aberrant product.

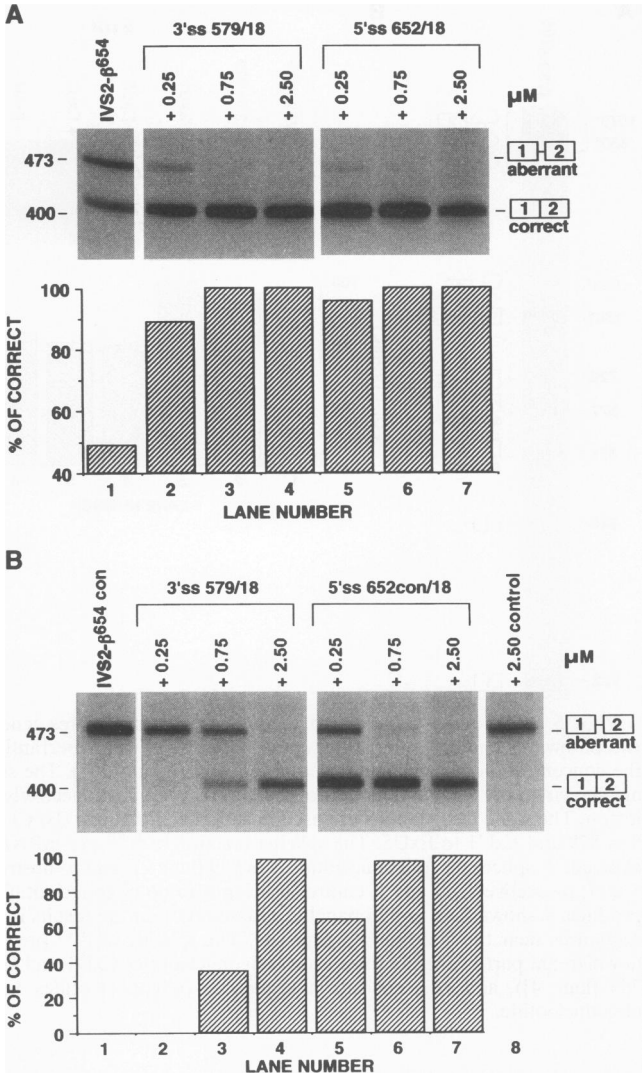

FIG. 8. Effects of 18-mer oligonucleotides on the splicing of β^{654} (A) and β^{654con} (B) pre-mRNAs. The splicing of β^{654} and β^{654con} pre-mRNAs was carried out in the presence of increasing concentrations of 18-mer oligonucleotides directed to the 3′ splice site at position 579 (lanes 2 to 4) and to the 5′ splice site at position 652 (lanes 5 to 7). Lanes 1, splicing without oligonucleotide. Panel B, lane 8, shows the splicing of β^{654con} pre-mRNA in the presence of noncomplementary oligonucleotide (control). The concentration of oligonucleotide is shown at the top of each lane. Quantitation of the results is shown below each autoradiogram.

(16). The length of about 50 nucleotides was also shown to be the lower limit for exons with weak splice sites recognizable in model pre-mRNAs both in vivo and in vitro (9, 51). The sequence analysis of the aberrant exon in β^{705} pre-mRNA did not reveal any AG dinucleotide or more than two purines in a row in the targeted regions (with the exception of the target for ineffective ExU5). This indicates that the strong inhibitory effect of 2′-*O*-methyl oligoribonucleotides could not be explained by the mechanism previously postulated for the splicing of β^{110} pre-mRNA (23, 54) or by the masking of purine-rich sequences shown to facilitate the inclusion of exons in a variety of pre-mRNA substrates (47, 49).

We conclude that the inhibitory effect of exon-targeted oligonucleotides reflects the amount of space needed for splicing factors to associate near the splice sites flanking the internal exon. The existence of such interactions is indeed supported by early experiments in which uncapped pre-mRNA was used as a splicing substrate (22, 37). These experiments showed that the region in human β-globin pre-mRNA extending approximately 20 nucleotides upstream from the exon 1 5′ splice site was protected against the activity of an endogenous 5′-to-3′ exonuclease (37). The factors likely to be displaced by the exon-targeted antisense oligonucleotides include U5 small nuclear RNA, shown to interact with exon nucleotides adjacent to both splice sites (33, 42, 50).

Experiments on the strength of 3′ and 5′ splice sites in β^{654} and β^{654con} pre-mRNAs led to the observation that the improvement of the 5′ splice site to match the consensus sequence did not significantly change its accessibility to antisense oligonucleotides but unexpectedly resulted in the decreased accessibility of the upstream 3′ splice site. Apparently, the improvement of the 5′ splice site gives rise to stronger interactions of splicing factors with the 3′ splice site, effectively displacing shorter antisense oligonucleotides targeted to this site. In fact, it has been shown that U2AF65 is recruited to the 3′ splice site by improved interactions of U1 small nuclear ribonucleoproteins at the downstream 5′ splice site through a network of interactions spanning the exon (18). Data from this work, demonstrating the existence of functional domains within exon sequences and measurable changes in the accessibility of flanking splice sites, provide additional support for the exon definition model, in which

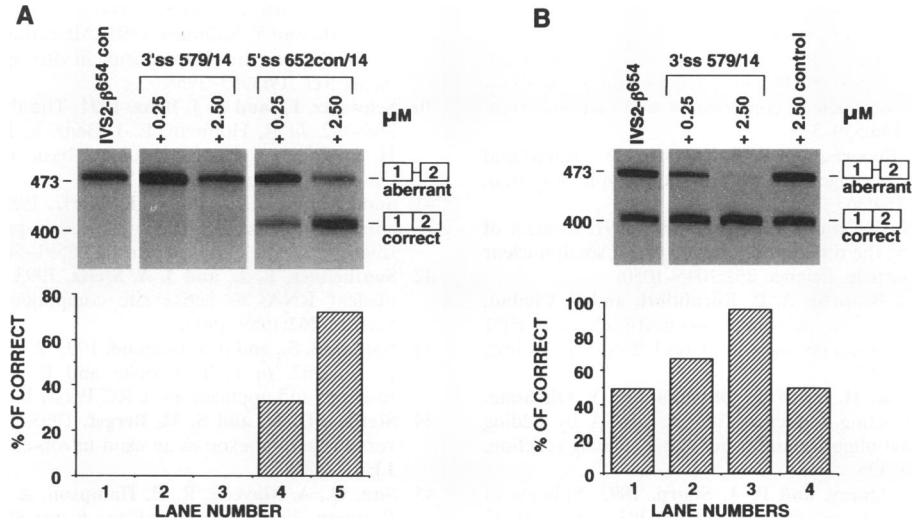

FIG. 9. Effects of 14-mer oligonucleotides on the splicing of β^{654con} (A) and β^{654} (B) pre-mRNAs. (A) The splicing of β^{654con} pre-mRNA was carried out in the presence of 14-mer oligonucleotides targeted to the 3′ cryptic splice site (lanes 2 and 3) and the consensus 5′ splice site at position 652 (lanes 4 and 5). Lane 1, splicing without oligonucleotide. (B) The splicing of β^{654} pre-mRNA was carried out in the presence of 14-mer oligonucleotide targeted to the 3′ cryptic splice site (lanes 2 and 3). Lane 1, splicing without oligonucleotide; lane 4, splicing with noncomplementary oligonucleotide (control). Quantitation of the results is shown below each autoradiogram.

exons, and not introns, are the units of assembly during spliceosome formation (36).

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